Appendix B (Application No.: 10/682584)

Teachings Of WO 01/27135 As A Whole and Obviousness/Unobviousness Analysis Of the Presently Claimed Invention In View Of WO 01/27135

The international patent application WO 01/27135 (Beachy'135) has largely similar or identical disclosure with the US 6432970 (Beachy et al'970) and US 7291626 (Beachy et al'626) analyzed before. Beachy'135 differs from Beachy et al'970 and Beachy et al'626 essentially in its further exemplifications (page 88, line 14 to page 133, line 18; Examples 1 to 3) and in its disclosure of compounds that "modulate cAMP levels" as additional subject compounds (page 47, line 22 to page 49, line 26). With regard to the disclosure of cAMP modulators as subject compounds, Beachy'135 is similar to Dudek et al. Therefore, concerning said disclosure, reference is made to the previously presented discussion of said matter in Dudek et al. Similarly, reference is made to the analyses of Beachy et al'970 and Beachy et al'626 that have been presented with regard to the disclosures of Beachy'135 that are identical or overlapping with them. In particular, it must be pointed out that the self-contradicting and ambiguous unsupported disclosures existing in Beachy et al'970 and in Beachy'626 exist also in Beachy'135. Also, Beachy'135 is similarly inoperative as Beachy et al'970 and Beachy et al'626 concerning treatments of entire categories of disorders enlisted therein as being treated beneficially by administration of an antagonist of Hedgehog signaling.

Beachy'135 teaches that its subject method of contacting a cell *in vitro* or *in vivo* with a Smoothened antagonist in an amount sufficient to antagonize the Smoothened-dependent activation of Hedgehog signaling (page 9, lines 22-25) may be used "*to treat conditions involving unwanted cell proliferation, e.g. cancer*" (page 10, lines 5-9). A Smoothened antagonist is defined on page 20, lines 5-13 as a compound which inhibits the Smoothened-dependent signaling initiated by contacting a cell with a Hedgehog protein. In Example 1 (described on page 10, line 16 to page 14, line 2 and page 88, line 14 to page 94, line 13), a cloned cell line of ptc -/- fibroblasts displaying constitutively activated Hedgehog/Smoothened signaling is described to show abolished Hedgehog/Smoothened signaling upon treatment with a sufficient amount of cyclopamine or a derivative thereof (KAAD cyclopamine) for two days (see Figure 4A). Notice that page 13 has a mix up and that the description starting on page 13, line 18 refers in reality to Figure 4A, not to 4B (see also Figures 4A and 4B on page 4/9). In the brief description of figures summarizing the experimental results of Example 1, Beachy'135 states that "*Significant toxicity (microscopically visible cell death, or decrease in Cell Titer reading) was not observed, even at the highest doses of compounds used*" (page 13, lines 31-32). Thus, Beachy'135 teaches that treatment of a cloned line of ptc -/- cells (which are transformed cells) with a Smoothened antagonist in an amount or dose that is sufficient to abolish the Smoothened-dependent Hedgehog signaling will not cause death of the treated cells. Notice in this regard that the Beachy'135 descriptions in Example 1 are largely similar with those in the Taipale et al, *Nature* 2000;406:1005-1009 publication that has been analyzed before. Concerning Example 1, therefore, reference is made further to the analysis of Taipale et al presented before.

Example 2 in Beachy'135 (page 94, line 15 to page 132, line 11) describes structure and synthesis of various compounds as well as their potency in inhibition of Hedgehog signaling in an *in vitro* screening assay. A compound derived from cyclopamine, 3-Keto, N-(N'-(N''-dihydrocinnamoyl aminocaproyl) aminoethyl) cyclopamine (abbreviated in Taipale et al as KAAD cyclopamine) is discussed in detail. It is stated that said derivative displaying greater potency than cyclopamine has the desired property of achieving inhibition of Shh signaling with much reduced toxicity (page 95, lines 11-17) and that said derivative and other more potent derivatives have broad utility in treatment of tumors (page 95, line 19 to page 96, line 6). Thus, concerning tumor treatment Beachy'135 has similar teaching as the Taipale et al publication analyzed before.

Example 3 (page 132, line 13 to page 133, line 18) is describing subcutaneous inoculation of fibroblast derived ptc -/- cells to nude mice to produce fibrosarcoma. Three weeks after inoculating five million ptc -/- cells, the subcutaneous tumor in each mouse was measured for length and width. Then one mouse was injected intraperitoneally with tomatidine in an unspecified dose, another was injected intraperitoneally with cyclopamine in an unspecified dose and a third mouse was similarly injected with KAAD cyclopamine in an unspecified dose. After four days of treatment, the animals were killed, the tumors were re-measured for length and width and the excised tumors were fixed and paraffin-embedded. Sections were examined by hematoxylene-eosin staining and by immunohistochemistry using a primary antibody to Ki-67, a proliferating cell marker. Results are depicted graphically in Figure 6 and it is stated that "proliferation rates" in these tumors were found to be 30%, 16% and 12% for the tumor from tomatidine, cyclopamine and KAAD cyclopamine injected nude mouse, respectively (page 133, lines 7-11). Whereas it is not stated explicitly, these percentages may be considered to be the frequency of Ki-67 labeled cells in each tumor. No statistics is given so it is not known how many microscopic fields or sections were evaluated with each tumor. However, presuming reproducibility, a 60% less proliferative activity can be calculated in the tumor of KAAD cyclopamine injected nude mouse relative to the tomatidine injected mouse and a 47% less proliferative activity can be calculated in the cyclopamine injected mouse.

Tumor volumes measured as the product of length and width (page 132, lines 20-21) are also shown in Figure 6 and stated to have increased 17% in the tomatidine injected mouse and decreased 0.14% and 19% in the cyclopamine injected mouse and KAAD cyclopamine injected mouse, respectively. Actual (absolute) sizes of the subcutaneously grafted tumors prior to and after intraperitoneal injections are not described.

Tumor grafts in nude (athymic) mice generated by subcutaneous inoculations of allogeneic mouse tumor cells or of xenogeneic human tumor cells have long been known in art. Subcutaneous inoculations of transformed fibroblasts of mice as well as of other rodents and of human to generate fibrosarcomas in nude mice have also been known. Therefore, a person skilled in the art knows the nude mouse model described in Beachy'135 and the sizes of subcutaneous fibrosarcomas and other tumors similarly generated in prior art. For example, the table below shows sizes of subcutaneous fibrosarcomas and related sarcomas described in prior art to be generated in nude mice by subcutaneous inoculation of five million transformed fibroblasts three weeks or more after inoculation. Tumor sizes are measured commonly using a caliper or by weighing excised tumors. To facilitate comparison with the commonly reported tumor diameter values, the results reported in mg tumor weight are converted to an average tumor diameter value in the table below using an approximation of a spherical tumor shape and indicated so.

Publication	Average tumor diameter (mm)	Time after inoculation	Number of nude mice
Jaye M et al, *EMBO Journal* 1998;7:963-969	~ 4 – 5	3 weeks	4
Ananthaswamy HN et al, *Cancer Research* 1988;48:3341-3346	~ 7 – 27	6 weeks	24
Lewis BC et al, *Cancer Research* 2000;60:6178-6183	~ 4 – 15	6 weeks	127
Wilson DM et al, *Cancer Research* 1990;50:5587-5593	6	19 – 36 days	13

Transformed fibroblasts of mouse (Jaye et al, Ananthaswamy et al), rat (Lewis et al) or human (Wilson et al) were inoculated subcutaneously to nude mice (five million cells per mouse, except in Jaye et al who inoculated 2.5 million cells). Tumor size is reported in mg in Lewis et al and converted to average diameter.

As described by the publications cited in Table 1 and in other prior art reporting other analyses of fibroblast-derived tumor grafts in nude mice (Auerbach R et al, *Cancer Research* 1978;38:1739-1744; Zietman AL et al, *Cancer Research* 1988;48:6510-6516; Taghian A et al, *Cancer Research* 1993;53:5012-5017), marked variability exists in tumor growth and phenotype from one nude mouse to another inoculated subcutaneously with the same number of same transformed fibroblasts. Marked variation between individuals of the same strain of nude mice is well known also with other subcutaneous tumor grafts (including carcinomas) and relates in part to the fact that nude mice, although immunocompromised, have residual T-lymphocytes and other immune effector cells that vary quantitatively and qualitatively from one nude mouse to another (see e.g. Zietman et al, *ibid*; Taghian et al, *ibid*). Whereas five million transformed cells inoculated subcutaneously produce varying size tumors, the average tumor size at week 3 is rather small (Table; see also Auerbach et al, *ibid*). A person skilled in the art knows that accuracy of tumor volume assessment requires measurement of a tumor in three dimensions rather than two and, in any case, even a 1 mm inaccuracy in measurement can give a 19% or greater variation with such small tumors.

Subcutaneous mouse tumors in nude mice are well known to have major differences from human tumors due in part to the residual immune response capability of nude mouse to foreign tumor cells and also due to the major differences between the rodent and human species, e.g. in genetic stability. In particular a large proportion of the cells in a subcutaneous tumor in nude mice, varying in proportion from one third to more than half of the cells, are non-tumorigenic immune system cells of the host invading the mass of foreign tumor cells (Zietman et al, *ibid*). A subtype of these tumor-invading host cells, macrophages, are found to consistently invade mouse tumors (including fibrosarcoma) and their content in mouse tumors is reported to correlate inversely with tumor size (Bucana CD et al, *American Journal of Pathology* 1992;141:1225-1236). Thus, a person skilled in the art knows that nude mice have residual T-lymphocytes and other immune effector cells that are well known to be capable of exerting anti-tumor response and that such cells, varying in quantity and quality from one nude mouse to another, do invade subcutaneous tumor grafts. Hence, it is clear to him or her that any effect on the non-tumorigenic host cells could also cause a 19% or greater variation in tumor size. This shortcoming of the nude mouse model, particularly with subcutaneous grafts, was well recognized in prior art. See the conclusion of Zietman et al, *ibid*, : "*There are ... serious difficulties with the model. An important one is the uncertain impact on the observed tumor response by the low level immune reactivity of nude mice. An optimal model for experimental therapeutics would have either no or a weak but well defined residual immune reactivity against tumor*". See Taghian et al (*ibid*) for a similar conclusion who, like Zietman et al, used immunosuppression to decrease the anti-tumor responses varying from one nude mouse to another and used also a more severely immunosuppressed mouse model, the SCID mouse.

Subcutaneous grafting of tumor cells, though convenient, was known to have shortcomings also due to other, non-immune, limitations (see Zietman et al, *ibid*; Kampschmidt RF et al, *Cancer Research* 1968;28:1938-1943). For example, Kampschmidt et al (*ibid*) had described spontaneous regression of a defined sarcoma due to the necrotic death of tumor cells when the tumor size reached to a limit even though the same sarcoma cells grafted to rectus femoris grew continuously to kill the host. See Lewis et al (*ibid*) for a similar description of the spontaneous necrotic death of fibrosarcoma cells grafted subcutaneously.

Coefficient of variation (variation expressed as percentage of mean) and the 95% confidence limit are quantitative measures of variation. The coefficient of variation (CV) values calculated from the statistical analyses described in prior art for tumor size variation from one nude mouse to another following subcutaneous inoculation with an identical number of same transformed cells are in the range of 23% (Lewis et al, *ibid*; average of observations) to 35% (Auerbach et al, *ibid*; with Ha/ICR strain nude mice), 59% (Auerbach et al, *ibid*; with BALB/c strain nude mice) and 61% (Jaye et al, *ibid*). Similarly, Zietman et al (*ibid*) and Taghian et al (*ibid*) described large variation in tumor growth and phenotype between the nude mice grafted subcutaneously with the same transformed fibroblasts (*several folds* difference from the lower to the upper end of 95% confidence limits). Auerbach et al (ibid) described further that anatomic site of subcutaneous inoculation of tumor cells must be strictly standardized since it has a large impact on tumor growth and that there is marked variation from one nude mouse to another even when the site of inoculation is standardized. A person skilled in the art, aware of aforementioned prior art, would know that a 19% variation in a single nude mouse is well within the variation described in prior art with subcutaneously grafted nude mice and that, furthermore, it cannot be taken to mean an elimination of tumorigenic cells from the mouse.

Thus, for multiple reasons Beachy'135 does not provide a reasonable basis for a person skilled in the art to contemplate the presently claimed invention (let alone to achieve it). Rather, the explicit Beachy'135 teachings mentioned before, including the teaching that treatment of a clonal line of ptc -/- fibroblasts with cyclopamine or with KAAD-cyclopamine does not cause death of said tumorigenic cells at the highest doses of compounds abolishing Hedgehog/Smoothened signaling (page 13, lines 31-32), lead away from the presently claimed invention.

Beachy'135 shows also microscopic views of hematoxylin-eosin stained tissue sections of the aforementioned subcutaneous tumors of nude mice (Figure 7). Bottom row in Figure 7 have two high magnification views, one labeled "Less differentiated" and the other "More differentiated". Arrows originating from the upper row section labeled "Tomatidine" as well as from the section labeled "KAAD-cyclopamine" are pointed at the same tissue section below labeled "More differentiated". As the same tissue section cannot belong to two different tumors, said figure is ambiguous. Since zonal variation in differentiation from one region of a tumor to another can occur, demonstration of an effect on differentiation would require statistical analyses of adequate number of tumor regions. Nevertheless, Beachy'135 states on page 133, lines 14-18 that the cyclopamine and KAAD cyclopamine treated tumor showed a greater degree of differentiation than the tomatidine treated tumor. It must be pointed out in this respect that whereas Beachy did analyze the hematoxylin-eosin stained tumor sections under high microscopic magnification (Figure 7, bottom row), there is no mentioning of a causation of tumor cell apoptosis. Such analyses of tumor sections would ordinarily allow detection of tumor cell apoptosis had it been caused (see DeJong JS et al, *British Journal of Cancer* 2000;82:368-373; notice also the use of microscopic analyses of hematoxylin-eosin stained tumor sections in the present application for detection of apoptosis). The present claim 47 recites inductions of both differentiation and apoptosis of tumor cells in the same tumor. It is also clear that induction of apoptosis of a particular tumor cell requires a dose of cyclopamine that is greater than a dose sufficing to induce differentiation of the same tumor cell (Appendix A).

Finally, a further fact, well known in art and relevant to an obviousness analysis, should also be pointed out: the vast majority of candidate cancer therapies, even when shown to be effective on a tumor by adequate testing in animal models, fail in human patients both due to the problems of animal models and due to the significant differences between the human and rodent species in the responses of their normal and neoplastic cells to various chemotherapy candidates. The presently described and claimed tumor treatment is based on the identification of a fundamental difference of tumor cells from normal cells and it is clear that it is not made obvious by the prior art contemplating and describing administration of a selective inhibitor of Hh/Smo signaling for inhibition of tumor cell proliferation (see the 6.11.2007 dated amendment).